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Effect of Disuse on Muscle Energy Metabolism Studied by *in vivo* 31-Phosphorus Magnetic Resonance Spectroscopy

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ABSTRACT—Chronic unloading induces disuse atrophy in rat hindlimb muscles. The purpose of this study was to examine the effect of hindlimb suspension on energy metabolism in hindlimb muscles during tetanic contraction using 31-phosphorus magnetic resonance spectroscopy (^{31}P-MRS). Muscular contraction was induced by electrical stimulation of the sciatic nerve at 40 Hz. Energy metabolism was evaluated by measuring the relative intensities of phosphocreatine (PCr), inorganic phosphate (Pi), β-ATP and phosphomonoester (PME). The intracellular pH was also estimated from the chemical shift of Pi. Two weeks of hindlimb suspension induced a greater weight loss in the soleus compared with the gastrocnemius muscle. The maximum tension at 40 Hz was significantly lower ($P<0.05$) in the hindlimb suspended group than in the control group. However, fatigability did not differ significantly. The energy level at rest was similar in the two groups. During electrical stimulation, the Pi/(Pi+PCr) ratio was higher in the hindlimb suspended group compared with the control group significantly ($P<0.01$), but the intracellular pH did not differ. These results suggest that the metabolic capacity of hindlimb muscle decreases after a prolonged period of disuse.

INTRODUCTION

Disuse atrophy of skeletal muscle has been studied in several experimental models, including small cage restraint [1], spinal cord transection [2], denervation [3, 4], tenotomy [3, 5], cast immobilization [6, 7], joint fixation [8] or paralysis with tetrodotoxin [9]. In 1979, the hindlimb suspension model was developed by Morey to simulate the influence of weightlessness [10]. With the hindlimb suspension model, in contrast to previous models, the hindlimbs are made completely non-weight bearing and the animal is able to both contract and relax the hindlimb muscle freely. Numerous investigators have begun to use this model to examine the effect of unloading on skeletal muscle and study the mechanism by which disuse atrophy is undergone.

The changes which occur in the suspended hindlimb have been studied histochemically. The percentage of slow-twitch fibers decreases in the soleus after the hindlimb is suspended [11, 12], while no such changes was observed in the gastrocnemius, tibialis anterior [13], and extensor digitorum longus muscles [12]. Significant changes in contractile properties, therefore, may be due to changes in the fiber composition of an unloaded muscle.

Histochemical and biochemical enzyme assays have shown that both anaerobic and aerobic capacity increase in slow-twitch muscle [11–13] while the aerobic capacity in fast-twitch muscles decreases during hindlimb suspension [13]. These studies were based on measurements made *in vitro* of isolated muscles. However, it is best to measure the enzymatic adaptation which affects muscle energy metabolism during contraction *in vivo*.

31-phosphorus magnetic resonance spectroscopy is being used to study the energy metabolism and mitochondrial function of skeletal muscle [14, 15]. This technique permits non-invasive and repetitive measurement of high energy phosphate, inorganic phosphate, and sugar phosphate level, as well as

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estimation of the intracellular pH. The purpose of this study was to examine the effect of prolonged muscle unloading on energy metabolism *in vivo* during muscular contraction.

MATERIALS AND METHODS

Animal care Male Wistar Kyoto rats (20–24 weeks) were used in this study. Temperature (25°C) and light-dark cycles (12 : 12 h) were maintained throughout the experimental period. Experimental rats were divided randomly into the control and hindlimb suspended (HS) groups.

Hindlimb suspension procedure The suspension method used in this study was a modification of Morey's tail-suspension model [10]. After the rat was anesthetized with pentobarbital sodium (50 mg/kg body weight), 1.2 mm diameter holes were drilled carefully through the third and fourth tail bones without disturbing blood flow to the tail. A stainless steel wire was inserted and attached to the top of the cage by a ball-bearing wheel swivel which allows 360° rotation. The suspension height was adjusted to prevent the hindlimbs from contacting the floor or the sides of the cage while the forelimbs were allowed to support their weight and to obtain food and water ad libitum.

Experimental preparation and muscle stimulation After 2 weeks of suspension, the rat was anesthetized with pentobarbital sodium (50 mg/kg body weight). The right sciatic nerve was exposed in the gluteal region and placed in contact with a small bipolar electrode. The right hindlimb was placed in a 2 cm diameter solenoid coil and was fixed on a small platform at both the knee and ankle with full extended position. The distal tendon of the gastrocmenius-plantaris-soleus muscle group was exposed at the ankle, cut, and attached to a strain gauge (TB611, NIHON KOHDEN) with a noncompliant thread, the output of which was displayed on a Polygraphy system (RM-6000, NIHON KOHDEN). The rat was inserted in the magnet. The hindlimb muscles were stimulated electrically (SEN-3301, NIHON KOHDEN) through an electrode with a 0.5 ms square wave pulse at 40 Hz for 20 min. Stimulation was performed with intermittent trains of 1 sec in duration, repeated every 2 sec. Stimulation voltage was 4 V which was supramaximal in both the control and HS groups.

31-phosphorus magnetic resonance spectroscopy (^{31}P-MRS) ^{31}P-MRS spectra were recorded with a BEM 250/80 spectrometer (OTSUKA ELECTRONICS U.S.A. INC.) operating at 32.3 MHz. The spectrometer was operated in the Fourier transform mode with a pulse duration of 15 μs (90°) and a pulse interval of 2 sec. Each spectrum was averaged over 2 min, which was from 60 free induction decays (FIDs). The phosphocreatine peak (PCr) was assigned as a chemical shift of zero. The levels of tissue PCr, inorganic phosphate (Pi), β-ATP and phosphomonoester (PME) were estimated from the areas under individual peaks. The cellular energy level was evaluated by the Pi/(Pi+PCr) ratio and the relative concentration of Pi, PCr, ATP, and PME were calculated as fractions of the total ^{31}P-MRS signal. Pulse interval of 2 sec reduced the PCr and Pi amplitudes to about 60% of their respective maximum. These saturation factor were incorporated into the calculation of relative concentration. The intracellular pH was estimated from the chemical shift of Pi relative to the PCr peak (δ) and calculated as follows [16]:

$$\text{Intracellular pH} = 6.90 - \{(\delta - 6.81)/(3.29 - \delta)\}$$

Measurement of muscle weight At the end of the experiment the rat was killed with an overdose of pentobarbital sodium. The hindlimb muscles were removed, cleaned of connective tissue, and weighed. Weights were expressed in absolute (mg) or relative (mg/g body weight).

Data analysis Values were expressed as mean ±S.E. Differences were tested for significance by Student's *t*-test.

RESULTS

The absolute and relative muscle weights from the control and the HS groups are shown in Table 1. They were significantly lower in the HS group than in the control group. The muscle mass loss

Table 1. Effect of hindlimb suspension on body weight, muscle weight and muscle weight to body weight ratios

	Control (6)	hindlimb suspension (6)	%change
BW(g)	378.0±3.1	349.0±5.0	− 5.7**
MW(mg)			
soleus	170.8±4.2	104.4±3.6	−38.9**
plantaris	421.3±5.6	350.5±4.0	−16.8**
gastrocnemius	1991.7±47.2	1557.6±53.6	−18.5**
MW/BW(mg/g)			
soleus	0.448±0.009	0.297±0.013	−33.7**
plantaris	1.111±0.014	1.006±0.021	− 9.5**
gastrocnemius	5.020±0.136	4.462±0.128	−11.1*

Values are means±S.E. The number of rats in each group is given in parentheses. BW, body weight; MW, muscle weight; MW/BW, muscle weight to body weighg ratios. Significant differences between control and hindlimb suspension groups are indicated by *($P<0.05$) and ** ($P<0.01$).

Table 2. Effect of hindlimb suspension on contractile properties in the soleus-plantaris-gastrocnemius muscle group

	control (6)	hindlimb suspension (6)	%change
P_o(g)	413.6±5.9	336.5±13.7	−18.6*
P_o/BW	167.1±1.1	167.0±1.5	

Values are means±S.E. The number of rats is given in parentheses. Abbreviations: P_o, maximum tension at 40 Hz; BW, body weight. A significant difference between control and hindlimb suspension is indicated by * ($P<0.05$).

was greater in the slow-twitch soleus than in the predominantly fast-twitch plantaris and gastrocnemius muscles.

The maximum tension at 40 Hz in the control and HS groups were 413.6±5.9 g and 336.6±13.7 g, respectively (Table 2). It was significantly lower in the HS group when compared with the control group ($P<0.01$). However the tension per gram of muscle did not differ between the control and HS groups. The percent decline in tension (% tension) 2 min after the onset of electrical stimulation

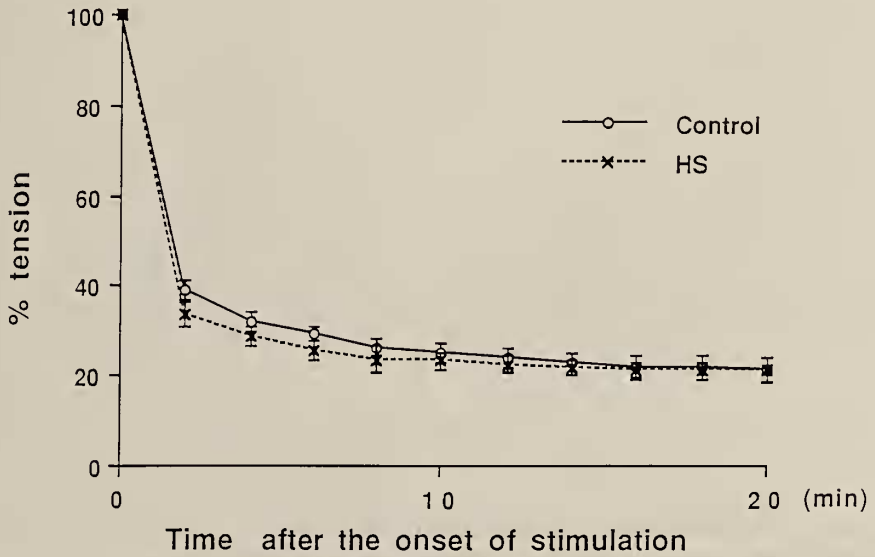

Fig. 1. Time course of changes in the % tension during electrical stimulation. HS, hindlimb suspension.

was 39.1±2.0% in the control group and 33.6± 2.3% in the HS group. At the end of the stimulation % tensions were 21.3±0.2 and 21.1±0.2%, respectively. There was no significant difference in the % tension decline between the two groups (Fig. 1).

The relative signal intensities of PCr, ATP, Pi, PME and the intracellular pH did not differ significantly at rest between the control and HS groups (Table 3).

Typical ^{31}P-MRS spectra taken at rest, during electrical stimulation, and during recovery are shown in Figure 2. Decreases in the PCr peak and increases in the Pi peak were observed during electrical stimulation. A lower level of PCr peak and higher level of Pi peak were maintained in the HS group when compared with the control group. After 10 min of recovery, the Pi and PCr peaks in both groups returned to the resting values.

Table 3. Relative signal intensities at rest of phosphomonoester (PME), inorganic phosphate (Pi), phosphocreatine (PCr), adenosine triphsphate (ATP) and intracellular pH in control and hindlimb suspension

	control (6)	hindlimb suspension (6)
PME	4.25±0.28	3.43±0.41
Pi	4.03±0.28	4.28±0.22
PCr	71.93±0.45	72.42±0.39
β-ATP	20.67±0.72	19.87±0.76
intracellular pH	7.08±0.03	7.08±0.03

Values are means±S.E. The number of rats is given in parentheses.

The time course of changes in the Pi/(Pi+PCr) ratio is shown in Figure 3(A). In the first 2 min of the stimulation, the Pi/(Pi+PCr) ratio in the control and HS groups increased to 0.71±0.03 and 0.84±0.02, respectively. After that, they returned gradually with time and were 0.55±0.04 and 0.74 ±0.03 at the end of the stimulation. The Pi/(Pi+ PCr) ratio was significantly higher in the HS group than in the control group during the stimulation. During the first 8 min of recovery, the Pi/(Pi+ PCr) ratio in both groups decreased rapidly and returned to the resting values without significant difference.

The time course of changes in the intracellular pH is shown in Figure 3(B). After the onset of the stimulation, the pH decreased and reached its lowest level within 2 min (6.36±0.02 in the control group and 6.45±0.03 in the HS group, respectively). From 2 min to the end of the stimulation, the intracellular pH increased gradually and returned

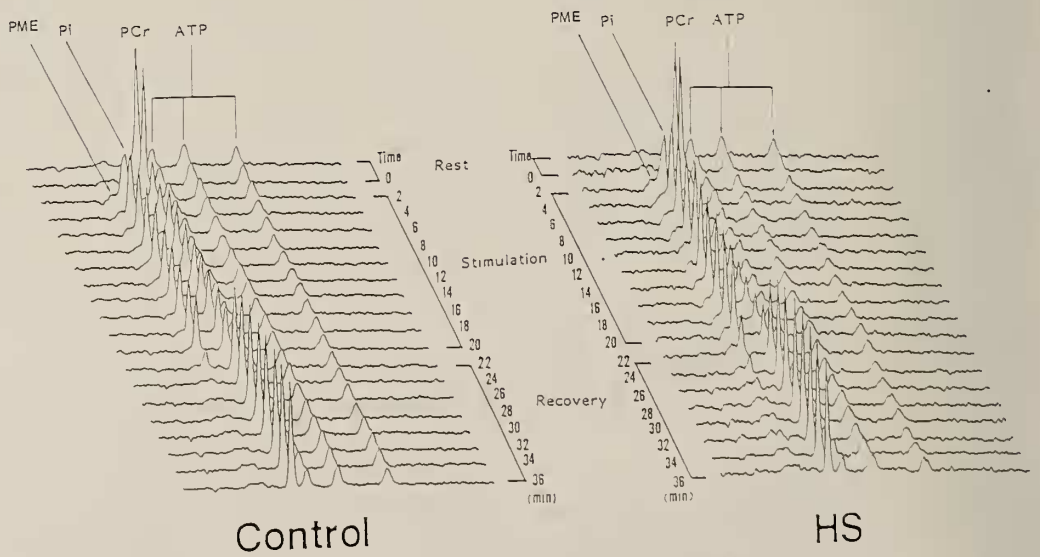

Fig. 2. A typical set of ^{31}P-MRS spectra at rest, during stimulation, and during recovery. HS, hindlimb suspension; PME, phosphomomoester; Pi, inorganic phosphate; and PCr, phosphocreatine.

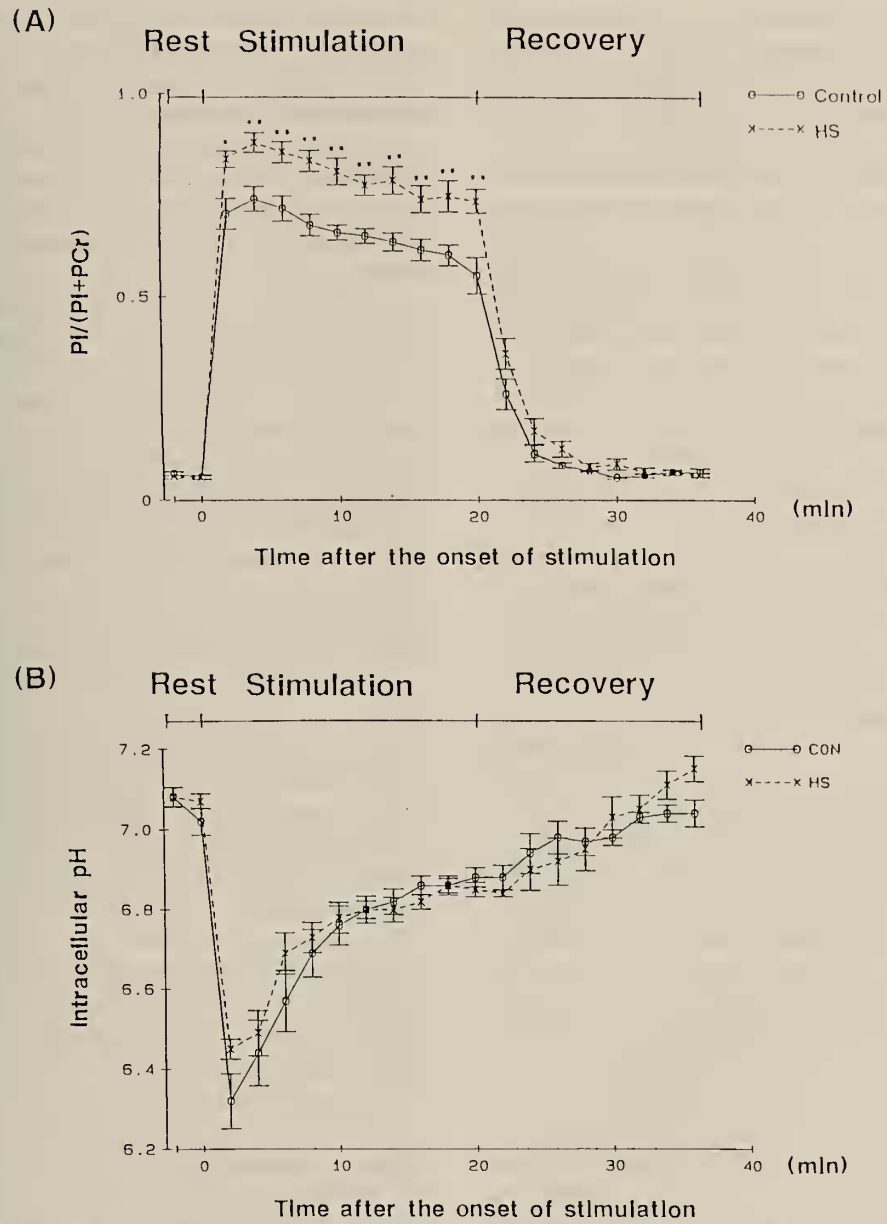

FIG. 3. Time course of changes in the Pi/(Pi+PCr) ratio (A) and the intracellular pH (B) at rest, during stimulation, and during recovery. Values are means±S.E. The number of rats in 6 in each group. HS, hindlimb suspension; Pi, inorganic phosphate; and PCr, phosphocreatine. Significant differences between control and HS groups are represented by * ($P<0.05$) and ** ($P<0.01$).

to the resting value within 10 min of recovery. There was no significant difference in the intracellular pH between the two groups throughout the experimental period.

DISCUSSION

Chronic unloading induces disuse atrophy in rat hindlimb muscles but the degree of muscle atrophy varies in which the slow-twitch muscle is more

susceptible to unloading that fast-twitch muscle. In the present study, the decrease of wet weight was greater for the soleus muscle than for the gastrocnemius muscle. These results are consistent with those of previous studies [13, 17–19].

A singificant decrease in the maximum tension was demonstrated after 2 weeks of suspension. The control and HS groups were similar with respect to the tension developed per gram of muscle. These results indicate that muscle atrophy may occur without selective myofibrillar protein loss during hindlimb suspension. The percent decline in tension development did not differ significantly between the two groups. In contrast, Fell *et al.* [20] reported faster rates of fatigue in gastrocnemius muscle after 1 week of suspension. This discrepancy might be due to differences in the stimulation parameters. Fitts and Hollozsy [21] reported that the degree of fatigue measured during a period of stimulation varied with the stimulation parameters used for the measurements. The stimulation parametes that we used induced contraction at a higher intensity than did those used by Fell *et al.* In suspended rat the gastrocnemius muscle, therefore, is thought to be more fatigable during stimulation at a lower inensity.

Chronic unloading affects musclar energy state as well as mechanical properties in the hindlimb muscle. Biochemical study of high energy phosphate concentrations in atrophied muscles reported that the concentrations of PCr and ATP were unchanged in the soleus while a significant reduction in ATP concentrations was noted in the extensor digitorum longus muscle [12]. In contrast, Jaspers *et al.* [24] reported that the ATP concentration increases in the soleus muscle after 6 days of tail-cast suspension, although they did not examine that in fast-twitch muscles. However, the ATP concentration relative to total adenosine nucleotide was unchanged in both the soleus and the extensor digitorum longus muscle [12]. In this study, relative concentrations of high energy phosphates were calculated for the estimation of energy state. At rest, high energy phosphates did not differ significantly between the control and HS groups. This result agrees with *in vitro* assay. There are some ^{31}P-MRS studies of energy levels in skeletal muscle during disuse. Zochodne *et al.* [22] reported that the patients with denervated muscles had a lower PCr concentration and a higher intracellular pH than did healthy control resting muscles. In contrast, the patients who had cast immobilization did not exhibit these changes. This difference is likely due to the type of disuse. Denervation removes trophic neural influence, by which the metabolic properties in skeletal may be affected.

High energy phosphates during electrical stimulation was significantly lower in the HS group than in the control group, whereas no difference was observed in the intracellular pH during electrical stimulation. Similar phenomena were observed in the patients with mitochondrial myopathy [23, 24]. In the patients with mitochondrial myopathy, the PCr concentration decreases to low values during exercise but without an accompaning severe intracellular acidosis in contrast to the observation in controls. Since free ADP is believed to be the principal driving force of mitochondrial respiration in skeletal muscle, the increase Pi concentration and the decreased PCr concentration suggest that there exists the elevated ADP concentration due to poor coupled oxidative phosphorylation in suspended hindlimb muscle. These results suggest that the decrease in aerobic capacity after hindlimb suspension induced low energy level during electrical stimulation.

The enzymatic adaptation of rat hindlimb muscle after hindlimb suspension has been reported [11, 13]. It is suggested that the capacity for glycolysis increases in slow-twitch and fast-twitch oxidative glycolytic fibers while fast-twitch glycolytic fibers are affected minimally. Aerobic capacity is reported to increase in slow-twitch fibers but decreases in fast twitch fibers. The result obtained by ^{31}P-MRS are consistent with the enzymatic changes in fast-twitch fibers during hindlimb suspension.

However, enzyme activities may not be the only factor to muscular energy metabolism *in vivo*. Oxygen delivery to the hindlimb muscle also affects the energy metabolism during musclar contractions. Since the ratio of capillaries per fiber in the soleus decreases 46% [12], oxygen delivery may also decrease. Sogabe *et al.* [25] reported that low inspired oxygen caused low energy level dur-

ing muscular contraction without intracellular acidosis. Both changes in enzyme activites and oxygen delivery may induce lower energy state in suspended hindlimb muscle during contraction.

While further study is required to assess the mechanism of metabolic adaptation during unloading of the hindlimb by the suspension, the present study demonstrates that ^{31}P-MRS may be used to assess the biochemical changes which occur in skeletal muscle under a variety of pathological conditions.

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